Occurrence and spread of multiresistant *Salmonella* Typhimurium DT104 in Danish animal herds investigated by the use of DNA typing and spatio-temporal analysis

M. N. SKOV[1]*, J. S. ANDERSEN[2] AND D. L. BAGGESEN[1]

[1] *National Food Institute, The Technical University of Denmark, Copenhagen, Denmark*
[2] *National Food Institute, The Technical University of Denmark, Søborg, Denmark*

SUMMARY

In the investigation we report the occurrence and spread of multiresistant *Salmonella* Typhimurium phage type (DT) 104 in 174 known Danish animal herds infected between 1996 and 2003. We applied PFGE, plasmid analysis and spatio-temporal analysis for a description of the development and spread of infection among herds. The results showed that in Denmark DT104 are mainly spread by trade of animals; however, horizontal spread of DT104 is also important and the risk of an animal herd becoming infected increased with the proximity in time and space to a DT104-infected herd. Based on these results, it is recommended that specific actions are taken to limit the risk of horizontal spread of DT104 from newly infected herds.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a causative agent of gastrointestinal salmonellosis in humans. Since its emergence in 1984 [1] *S.* Typhimurium phage type [definitive type (DT)] 104 has been isolated worldwide [2]. In Denmark, the United Kingdom and Germany it is one of the most common causes of human salmonellosis [3, 4]. It is in general characterized by resistance to at least five different antimicrobial agents: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. The multidrug resistance of DT104 combined with its ability to acquire resistance genes [5] and spread throughout the world [2] have generated the need to control this organism.

Since foods of animal origin are known to be an important source of these infections in humans [6], the presence of multiresistant (MR) *S.* Typhimurium DT104 in food is considered unacceptable by animal producer organizations, consumers, and by the veterinary authorities in Denmark. Therefore, the Danish *Salmonella* control and surveillance system has been updated and modified to identify infected animal herds [3, 7]. However, the system is primarily based on tracing the source of infections through the trade of animals, which leaves the source of a number of infections unknown and might overlook horizontal spread as a source of infection [8, 9].

In the present study spatio-temporal analysis has been used in combination with studies of the clonality of bacteria to study the spread of infection. Thus, data on time of detection of infection, geographical location, trade contacts, and DNA type of DT104 isolates from each of the infected herds have been included in the analysis. Based on the results, the mode of spread and the relevance of horizontal spread in the epidemiology of DT104 infections are discussed. To our knowledge the present paper offers the first example of the combined use of

* Author for correspondence: M. N. Skov, D.V.M., Ph.D., Department for Clinical Microbiology, Odense University Hospital, J.B. Winsløws Vej 21, DK-5000 Odense C, Denmark.
(Email: Marianne.Skov@ouh.regionsyddanmark.dk)

Table. The *distribution of DNA types* among pig herds, cattle herds, combined pig and cattle herds, and others during the study period from 1996 to January 2003*

PFGE, plasmid and DNA types				No. of isolates per year								No. of isolates per animal species			
*Bln*I	*Xba*I	Plasmid	DNA type	96	97	98	99	00	01	02	Total	Others	Cattle	Cattle & pigs	Pigs
B1	X1	95	I	3		8	9	34	24	11	89	2	7	5	75
B1	X1	95; 2·2	II	1	1	3	9	10	2	7	33		11	2	20
B2	X1	95	III		1						1			1	
B1	X1	95; 5	IV		1						1				1
B1	X1	95; 2	V			6			1	1	8	2			6
B1	X1	95; 5; 3·5	VI		1						1				1
B1	X2	95	VII		1						1				1
B1	X1	95; 3·5	VIII			1	1				2				2
B1	X1	95; 3·5; 2·2	IX			2	1	1	2		6	1			5
B1	X3	95; 2·2	X				1				1		1		
B3	X4	95	XI					2			2				2
B1	X1	95; 35; 10·5	XII					1			1				1
B1	X1	95; 35	XIII			1		9	6	1	17		3	2	12
B4	X1	95; 2·2	XIV							1	1				1
B5	X5	95	XV						4		4				4
B6	X6	95	XVI						1		1				1
B7	X7	95	XVII						2		2				2
B1	X1	95; <7	XVIII							1	1				1
B1	X8	95; 2·2	XIX						1		1				1
B1	X8	95	XX						1		1				1
			Total	4	5	21	21	57	44	22	174	5	22	10	137

* A DNA type is based on the combined results from PFGE typing and plasmid analysis.

spatio-temporal analysis and DNA typing in describing the epidemiology and spread of DT104 among infected animal herds.

METHODS

All herds known to be infected with DT104 between the first introduction in July 1996 until January 2003 were included in the investigation [3]. The 174 herds included 137 pig herds, 22 cattle herds, 10 mixed pig and cattle herds, two turkey flocks, two broiler chicken flocks, and one fox herd. The herds were identified as DT104 positive through the Danish *Salmonella* control and surveillance programmes [3, 7] or after submission of material to the National Food Institute for diagnosis of clinical disease. Each herd was represented by one isolate (Table).

Bacterial isolates selected for typing

Isolates included in the present study originated from samples cultured at the National Food Institute or at approved laboratories with conventional culturing methods using a non-selective pre-enrichment step, followed by plating on the selective media [Modified Semisolid Rappaport–Vassiliadis (MSRV)] [10], and for clinical samples, an additional incubation step was done using cysteine-selenite broth.

Before being available for the present study the isolates were serotyped according to the Kaufmann–White serotyping scheme [11] and the definitive phage type was defined according to the phage-typing scheme described by Callow [12] and extended by Anderson *et al.* [13]. Only isolates being resistant to minimum ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline were included in the study. Until January 2000 the susceptibility to antimicrobial agents was determined by tablet diffusion as previously described [14]. From January 2000 susceptibility to antimicrobial agents was performed as minimum inhibitory concentration (MIC) determinations using a commercially prepared, dehydrated panel (Sensititre; TREK Diagnostic Systems, UK) following NCCLS guidelines [15].

Pulsed-field gel electrophoreses analysis (PFGE)

Genomic DNA was prepared in agarose plugs and PFGE was performed as described by Olsen *et al.* [16]. For restriction endonuclease digestion, a 2-mm square of the plug was placed in a microfuge tube with 1 μl restriction buffer supplied by the enzyme manufacturer. After 1 h equilibration at 37 °C, 30 U of *Bln*I (Amersham Biosciences, Hillerød, Denmark) or *Xba*I (Amersham Biosciences) was added and the bacterial DNA was digested for 4 h at 37 °C. The plugs were loaded into 1% agarose gel (Seakem LE agarose, Medinova Scientific, Glostrup, Denmark) that was prepared in the running buffer, 0·5× TBE [45 mM Tris, 45 mM boric acid, 1·0 mM EDTA (pH 8·0)]. Bacteriophage lambda (48·5 kb) concatemers (Bio-Rad, Copenhagen, Denmark) was used as size standards. The gels were run at 6 V/cm at 14 °C for 20 h in a CHEF Mapper (Bio-Rad). Pulse-times were ramped from 2 s to 64 s and a 120° reorientation angle was applied. The gels were stained with ethidium bromide (100 μg/l), destained in water and photographed under UV illumination. The gels were analysed manually and all bands including small bands were included in the manual interpretation of PFGE patterns. Strains were allocated to different PFGE types based on differences of one band and classified as belonging to the PFGE types B1–B7 and X1–X8, after digestion with *Bln*I and *Xba*I, respectively.

Plasmid profile analysis

Plasmid DNA was extracted by a modification of the method of Kado & Liu [17] as described by Olsen *et al.* [18] and visualized after electrophoresis at 50 V for 15 min. followed by 100 V for 2·5 h on vertical 0·8 % (w/v) Tris–borate agarose gel (SeaKem LE agarose, Medinova Scientific). Plasmids in *Escherichia coli* 39R861 [19] and V517 [6] served as plasmid size reference molecules and sizing of plasmids was performed, as described by Olsen *et al.* [18]. Strains were allocated to different plasmid profiles based on differences in the size and number of the plasmids the strain harboured.

Definition of DNA type

Based on differences in the *Bln*I–, *Xba*I–PFGE type or the plasmid profile, strains were assigned to theit respective DNA type (named I–XX). Consequently strains having a B1 *Bln*I–PFGE type, a X1

*Xba*I–PFGE type and harbouring a 94 kb plasmid were allocated to DNA type I, where as DNA type II was pseudonymous with strains having B1, X1 PFGE types but harboured a 94 kb and a 2·2 kb plasmid (see Table).

The term non-DNA type I is used for all other strains except DNA type I. Strains having a DNA type I were excluded in some of the statistical analyses, since DNA type I, being the most prevalent DNA type in the world, is know to harbour strains that are not epidemiologically associated.

Statistics

All analyses were done using S-plus 6.1 [20]. Based on the geographic coordinates [(x_i, y_i), $i \in (1, 2, …, 174)$] for each farm a map was drawn to describe the geographic relationship of the infected herds. The distances between all farms $\|((x_i, y_i), (x_j, y_j)\|$ were calculated as 'spherical distances' by the Haversine formula [21]. Furthermore time differences $|t_i - t_j|$ of detection of infections were calculated. The herds were stratified based on their relationship to an 'epidemiological cluster' (i.e. herds being epidemiologically associated to each other) and by DNA type.

For all herds within a stratum the distance in time and space to herds within the same stratum (only non-DNA type I, and limited to a period of 180 days) were calculated. One special stratum was defined as infected herds not belonging to an epidemiological cluster. If these herds were located randomly in time and space the distribution of distances to herds infected with the same DNA type should not differ from the distribution of distances to other herds infected with other DNA types. This hypothesis was visualized graphically and tested using a Wilcoxon rank sum test [22].

RESULTS

DNA typing results

Seven *Bln*I–PFGE types, eight *Xba*I–PFGE types, and 12 plasmid types (Table and Fig. 1 *a*, *b*) combined to produce a total of 20 mutually exclusive DNA types (Table). As seen in the Table DNA types I (51%) and II (19%) were the only DNA types that remained present throughout the study period. Eleven of the 20 DNA types were only represented by one isolate.
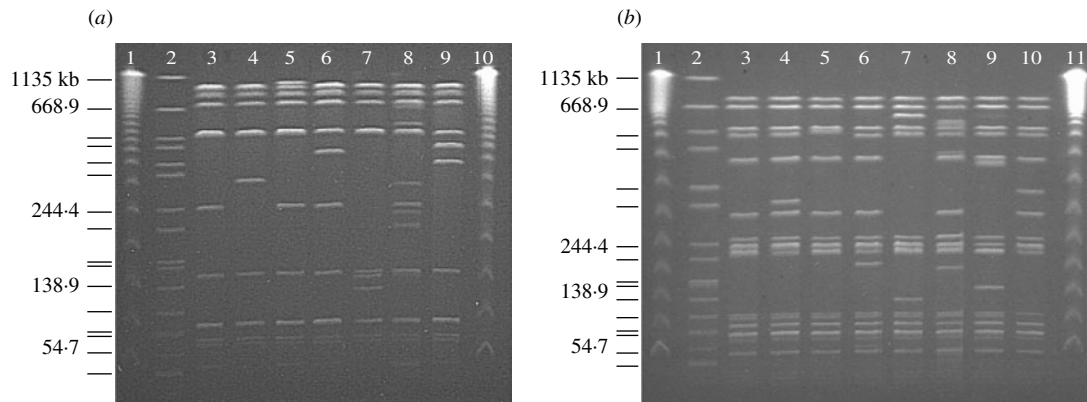
Fig. 1. PFGE types by the use of (*a*) *Bln*I and (*b*) *Xba*I. (*a*) Lane 1, size marker (lambda ladder); lane 2, CDC PFGE marker (digested by *Xba*I); lane 3, PFGE type B1; lane 4, PFGE type B2; lane 5, PFGE type B3; lane 6, PFGE type B4; lane 7, PFGE type B5; lane 8, PFGE type B6; lane 9, PFGE type B7; lane 10, lambda ladder. (*b*) Lane 1, size marker (lambda ladder); lane 2, CDC marker; lane 3, PFGE type X1; lane 4, PFGE type X2; lane 5, PFGE type X3; lane 6, PFGE type X4; lane 7, PFGE type X5; lane 8, PFGE type X6; lane 9, PFGE type X7; lane 10, PFGE type X8; lane 11, lambda ladder.

Descriptive epidemiology at herd level

Since the first case in July 1996, and until January 2003, 174 animal herds were found to be infected with DT104 in Denmark. The location and primary animal species of the herds are given in Figure 2. Based on records of epidemiological relationships (primarily trade contacts) 36 epidemiological clusters were identified, representing 67% of the infected herds. However, for the remaining 33% of the infected herds no record of contacts or sources of infection were recorded.

Spread of infection

By combining the DNA typing results with the geographical location and time of detection of infection, it was possible to compare the cumulative distribution of the distance between all herds, with the cumulative distribution of the distance between herds with no epidemiological relationship but having identical DNA types. The distributions included only infections with a time difference <6 months. If there were to be no transmission of DT104 between the herds with no epidemiological relationship, one would expect the cumulative distribution of the distance between these herds to be the same as for all herds. However, as seen in Figure 3, herds with no epidemiological relationship to other herds (referred to as 'non-trade-related herds' in the Figure), were located significantly (*P* < 0·001) closer to herds with the same DNA types than should have been expected from the overall distribution of distances between infected herds (referred to as 'All herds' in the Figure). In
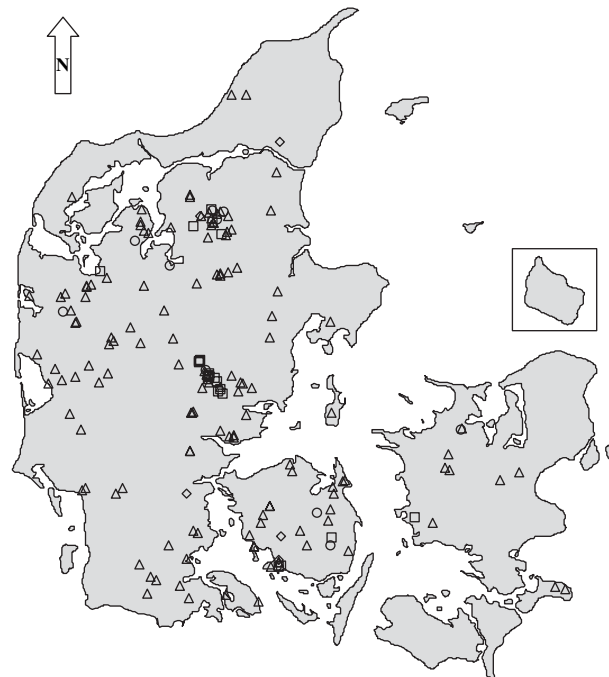

Fig. 2. The location of DT104-infected herds in Denmark 1996–2002. △, Pigs (*n* = 137); ○, pigs and cattle (*n* = 10); □, cattle (*n* = 22), ◇, other (*n* = 5).

Figure 3 it can be seen that 50% of the herds without an epidemiological relationship to other herds are located within a distance of 27 km from herds with the same (non-type I) DNA type. In total, 25% of these herds were located within a distance of about 8 km from herds with the same (non-type I) DNA type. Regarding all herds, 50% are located within a distance of about 95 km and 25% of all herds are within 70 km. No such relationship of shorter distances was
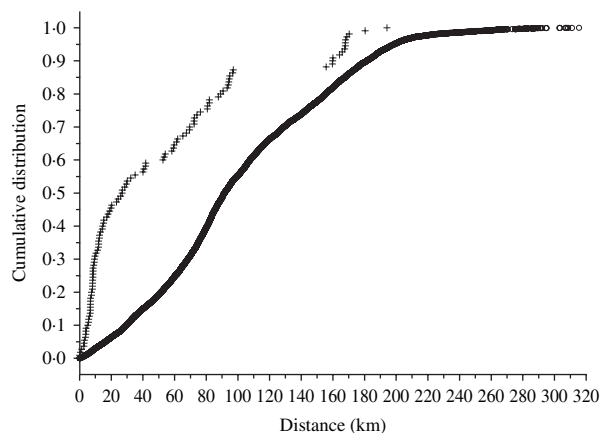
Fig. 3. The cumulative distribution of the distance between herds (for 'non-epidemiologically related herds' only herds infected within a time-frame of 6 months between the infections are included). ○, All herds; +, non-epidemiologically related herds.

seen when analysing non-epidemiologically related herds infected with DNA type I (data not shown).

DISCUSSION

The present study combines the use of DNA typing and spatio-temporal analysis for epidemiological investigations of DT104. By combining PFGE and plasmid results we identified a total of 20 DNA types. However, the majority of DT104 isolates displayed one of two dominant DNA types (types I and II), as these two DNA types included 70 % of all isolates. In the study strains were allocated to different PFGE types based on differences of one band. This is not in agreement with the criteria suggested by Tenover et al. [23]. However, as certain serotypes of Salmonella are known to be very homogenous groups of bacteria [2, 24] the use of the criteria of Tenover et al. [23] might result in even less discrimination. Furthermore, a substantial part of the discrimination in the present study is based upon plasmid profiling. Based on AFLP, PFGE, and plasmid typing other investigations have reached similar results, and it has been shown that the clonality of DT104 and certain phage types of S. Enteritidis may hamper molecular epidemiological investigations, due to lack of discrimination between epidemiologically related and non-related isolates [25, 26]. Although the inclusion of more isolates per herd might reveal further discrimination [8] the situation warrants combining disciplines in order to describe the epidemiology of the infection.

Our results showed that 67 % of the Danish DT104-infected herds had epidemiological relationships (primarily trade). These results, in addition to the identification of 36 epidemiological (primarily trade) clusters, supported the assumption that trade of animals is an important source of infection and might indicate that the Danish trace-back strategy [7] based on trade relations has effectively minimized the spread of DT104 between herds. On the other hand it was also shown that 33 % of the herds had no epidemiological relationship (trade or other known contact) to other infected herds. Although it is mandatory to report epidemiological contacts for DT104-infected herds in Denmark, it is not possible to exclude the possibility of a few unreported epidemiologically related herds still being included in the 33 %. Analysis of the geographic location and DNA typing results of these herds showed that the probability of becoming infected increased with the proximity in time and space to other infected herds. However, it is not possible to exclude selection bias in the study as we only included detected herds and there is the possibility of some herds going undetected. Sato et al. [27] have used spatial and temporal clustering to investigate the spread of Salmonella in dairy cattle in California, however, to our knowledge no specific risk distances have been described. The observed (time, distance) points in the present investigation are to some degree also uncertain as the registered time of infection may differ from the actual time of infection, therefore space–time regression was not applied. However, the overall results indicate that horizontal transmission may be important to the transmission of DT104 between herds, and investigation on vectors and vehicles for horizontal transmission therefore becomes important in order to improve the overall control of the spread of infection. Salmonella has been isolated from manure [28] and wild animals [29] and has in some cases been associated with the introduction of the infection at herd level [30]. Although a Danish investigation has shown that no Salmonella were found in pig manure after 2 weeks [28], others have found that purchase of animal manure poses a risk for the spread of Salmonella to other herds [31, 32].

In conclusion, the combined use of DNA typing and spatio-temporal analysis has enhanced our understanding of the transmission of DT104 between herds. In the investigation, the importance of the trade of animals in relation to the spread of DT104 between herds was shown, suggesting that the Danish

trace-back strategy together with ban on trade of animals from infected herds [7] has been important in reducing the spread of DT104 between herds. However, the analysis also enabled us to demonstrate the potential importance of horizontal spread of the bacteria and consequently the significance of fast elimination of the bacteria from infected herds. Overall, the presence of an infected herd constitutes a risk of the spread of infection to other nearby herds even though the mode of this horizontal transmission cannot be determined.

ACKNOWLEDGEMENTS

DECLARATION OF INTEREST

None.

REFERENCES

1. **Evans S, Davies R.** Case control study of multiple-resistant *Salmonella* typhimurium DT104 infection of cattle in Great Britain. *Veterinary Record* 1996; **139**: 557–558.

2. **Baggesen DL, Sandvang D, Aarestrup FM.** Characterization of *Salmonella enterica* serovar typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. *Journal of Clinical Microbiology* 2000; **38**: 1581–1586.

3. **Anon.** Annual report on zoonoses in Denmark 2002. Ministry of Food, Agriculture and Fisheries, 2003.

4. **Threlfall EJ, et al.** Increasing spectrum of resistance in multiresistant *Salmonella* typhimurium. *Lancet* 1996; **347**: 1053–1054.

5. **Carlson SA, Ferris KE.** Augmentation of antibiotic resistance in *Salmonella* typhimurium DT104 following exposure to penicillin derivates. *Veterinary Microbiology* 2000; **73**: 25–35.

6. **Macrina FL, et al.** A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* 1978; **1**: 417–420.

7. **Wegener HC, et al.** *Salmonella* control programs in Denmark. *Emerging Infectious Diseases* 2003; **9**: 774–780.

8. **Langvad B, et al.** Transmission routes of *Salmonella* Typhimurium DT 104 between 14 cattle and pig herds in Denmark demonstrated by molecular fingerprinting. *Journal of Applied Microbiology* 2006; **101**: 883–890.

9. **Rugbjerg H, et al.** Estimating the number of undetected multi-resistant *Salmonella* Typhimurium DT104 infected pig herds in Denmark. *Preventive Veterinary Medicine* 2004; **65**: 147–171.

10. **Stege H, et al.** Prevalence of subclinical *Salmonella enterica* infection in Danish finishing pig herds. *Preventive Veterinary Medicine* 2000; **44**: 175–188.

11. **Popoff MY, Le Minor L.** Antigenic formulars of the *Salmonella* serovars. Paris, France: WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, 1997.

12. **Callow BR.** A new phage-typing scheme for *Salmonella* typhimurium. *Journal of Hygiene (Cambridge)* 1959; **57**: 346–359.

13. **Anderson ES, et al.** Bacteriophage-typing designations of *Salmonella* typhimurium. *Journal of Hygiene* 1977; **78**: 297–300.

14. **Aarestrup FM, et al.** Resistance to antimicrobial agents used for animal therapy in pathogenic-, zoonotic- and indicator bacteria isolated from different food animals in Denmark: a baseline study for the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP). *APMIS* 1998; **106**: 745–770.

15. **National Committee for Clinical Laboratory Standards.** Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved Standard, 5th edn. NCCLS Document M7-A5, 2000.

16. **Olsen JE, et al.** Clonal lines of *Salmonella enterica* serotype Enteritidis documented by IS*200*-, ribo-, pulsed-field gel electrophoresis and RFLP typing. *Journal of Medical Microbiology* 1994; **40**: 15–22.

17. **Kado CI, Liu S.-T.** Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology* 1981; **145**: 1365–1373.

18. **Olsen JE, et al.** Plasmid profiles as an epidemiological marker in *Salmonella enterica* serovar *berta* infections. Comparison of isolates obtained from humans and poultry. *APMIS* 1992; **100**: 221–228.

19. **Threlfall EJ, et al.** Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of Salmonella typhimurium phage type 204c isolated in Britain. *Journal of Hygiene (London)* 1986; **97**: 419–426.

20. **S-PLUS 6.1 Professional.** Seattle, WA, USA: Insightful Corporation, 2002.

21. **Sinnot RW.** Virtues of the haversine. *Sky and Telescope* 1984; **68**: 159.

22. **Conover WJ.** *Practical Nonparametric Statistics*. New York: Wiley, 1980.

23. **Tenover FC, et al.** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* 1995; **33**: 2233–2239.

24. **Baggesen DL, Wegener HC, Madsen M.** Correlation of conversion of *Salmonella* enterica serovar enteritidis phage type 1, 4, or 6 to phage type 7 with loss of lipopolysaccharide. *Journal of Clinical Microbiology* 1997; **35**: 330–333.

25. **Lukinmaa S, et al.** *Salmonella* Enteritidis phage types 1 and 4: pheno- and genotypic epidemiology of recent

outbreak in Finland. *Journal of Clinical Microbiology* 1999; **37**: 2176–2182.

26. **Hilton AC, Penn CW.** Restriction enzyme analysis of randomly amplified polymorphic DNA amplicons of *Salmonella* enterica ser. Enteritidis PT4 and Typhimurium DT104. *Letters in Applied Microbiology* 1998; **27**: 158–162.

27. **Sato K, *et al.*** Spatial and temporal clustering of *Salmonella* serotypes isolated from adult diarrheic dairy cattle in California. *Journal of Veterinary and Diagnostic Investigation* 2001; **13**: 206–212.

28. **Boes J, *et al.*** Survival of *Escherichia coli* and *Salmonella* Typhimurium in slurry applied to clay soil on Danish swine farm. *Preventive Veterinary Medicine* 2005; **69**: 213–228.

29. **Liebana E, *et al.*** Molecular fingerprinting evidence of the contribution of wildlife vectors in the maintenance of *Salmonella* Enteritidis infection in layer farms. *Journal of Applied Microbiology* 2003; **94**: 1024–1029.

30. **Kirk JH, Holmberg CA, Jeffrey JS.** Prevalence of *Salmonella* spp. in selected birds captured on California dairies. *Journal of the American Veterinary Medical Association* 2002; **220**: 359–362.

31. **Warnick LD, *et al.*** Risk factors for clinical salmonellosis in Virginia, USA cattle herds. *Preventive Veterinary Medicine* 2001; **49**: 259–275.

32. **Veling J, *et al.*** Risk factors for clinical *Salmonella enterica* subsp. *enterica* serovar Typhimurium infection on Dutch dairy farms. *Preventive Veterinary Medicine* 2002; **54**: 157–168.